Immunostimulatory Capabilities of Highly Enriched Langerhans Cells In Vitro

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Langerhans cells (LC) are epidermal antigen-presenting cells capable of inducing allogenic, antigen-specific, and cytotoxic T cell proliferation. Previous studies have examined the dynamics of LC maintained in vitro in crude epidermal cell (EC) suspensions in which the major cell type is the keratinocyte (KC). To avoid the confounding effects of KC and other immunoregulatory cells on LC dynamics in vitro, highly enriched murine LC (85%) were studied, through 72 h of incubation in vitro, for their ability to present alloantigen (in a primary allogenic proliferation assay) and foreign antigen (in a secondary autologous proliferation assay). The results were compared to similar studies using crude EC suspensions. Freshly prepared LC are very poor stimulators of a primary allogenic proliferation response, with a 12- to 16-fold increase in stimulatory capacity by 72 h using panned-enriched and crude EC suspensions, respectively. Similarly, freshly prepared LC are weak stimulators of a secondary autologous proliferation response, with a 2.5- to 6-fold increase in immunostimulatory capability by 72 h. The overall increased stimulatory effect observed with the crude EC suspensions compared to highly enriched LC is most likely attributed to the effect of KC on T cell proliferation, rather than to a maturation effect of KC on LC during the 72 h of in vitro incubation. Using back-scattered electron imaging, the surface density of MHC-class II molecules (Ia) increased three- to fourfold through culture, which parallels the increase in functional ability. This study demonstrates that LC in either a crude or highly enriched cell suspension mature into potent immunostimulatory cells after incubation in vitro with an increased surface expression of Ia molecules. Keratinocytes are not necessary for LC maturation in vitro, but seem to exert some stimulatory effect by enhancing lymphocyte proliferation in the functional assay system. *(J Invest Dermatol 90:201–206, 1988)*

The Langerhans cell (LC), discovered by Paul Langerhans in 1868, is a migratory bone marrow–derived dendritic cell with distinctive intracytoplasmic Birbeck granules, comprising 3 to 8% of all epidermal cells [1]. It is the major accessory cell of the skin-associated lymphoid, capable of inducing allogenic, antigen-specific, and cytotoxic T cell proliferation [2–5]. The MHC-class II molecule, expressed by most LC, is the surface marker commonly used to study this cell type in the mouse.

Several studies have compared the functional dynamics and cellular characteristics of freshly prepared LC to LC maintained in vitro in short-term incubation [7–10]. Experimental evidence supports the fact that freshly prepared LC are relatively weak accessory cells, but become active stimulators of T cell proliferation after 2 to 3 days of incubation in vitro [7–8]. These previous studies, however, have examined the dynamics of LC in either a crude EC suspension or an EC suspension partially enriched for LC, in which the major cell type is still the keratinocyte. Although Inaba et al [8] examined the function of purified LC, the purification step occurred after the LC were co-cultured with keratinocytes.

Keratinocytes (KC) are immunologically active cells that may significantly affect maturation of LC or the results of functional assays employed in these previous studies. KC produce a number of cytokines that may enhance or inhibit lymphocyte proliferation, including interleukin-1 (ETAF), interleukin-2 (KTGF), and prostaglandins [11–14].

To avoid the possible confounding effects of KC and perhaps other immunoregulatory cells on LC dynamics, we studied highly enriched LC suspensions (85% LC) during 72 h of incubation in vitro for their ability to present alloantigen and foreign antigen to T lymphocytes in a Langerhans cell-lymphocyte proliferation assay. To determine the effect of other epidermal cell types (ie, keratinocytes) on LC maturation during short-term incubation in vitro, we compared the results obtained with highly enriched LC to those using crude EC suspensions. Additionally, we examined the

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Abbreviations:

BEI: back-scattered electron imaging
cMEM: complete minimum essential medium
CPM: counts per minute
EC: epidermal cell
ETAF:epidermal cell thymocyte-activating factor
FCS: fetal calf serum
GAM-G40: goat anti-mouse antibody conjugated to 40-nm colloid gold granules
GP: gold particles
KC: keratinocyte
KTGF: keratinocyte-derived T-cell growth factor
LC: Langerhans cell
MEM: minimum essential medium
MLR: mixed lymphocyte response
PA-G15: protein A-colloid gold granules (15 nm)
PBS: phosphate-buffered saline
SEM: scanning electron microscopy
SRBC: sheep red blood cells

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changes in surface density of Ia molecules by direct visualization using back-scattered electron imaging (BEI) and correlated this with functional ability.

MATERIALS AND METHODS

Media and Enzymes For enrichment of murine Langerhans cells, complete minimum essential medium (cMEM) was used: MEM (Gibco Laboratories, Grand Island, NY) supplemented with 25 mM HEPES buffer (Gibco), 100 U/ml penicillin G, 100 μg/ml streptomycin, 250 μg/ml amphotericin B (Fungizone^R, Gibco), and 200 mM L-glutamine. Heat-inactivated fetal calf serum (FCS) was added as indicated. Enzymes included neutral protease (Dispase^R, Grad II, Boehringer Mannheim, Indianapolis, Indiana), DNAse (bovine pancreas; Sigma Chemical Co., St. Louis, MO, D-0876), and trypsin (bovine pancreas; Sigma Chemical Co., T-2395).

Epidermal Cell Suspension Epidermal cell suspensions were obtained from the pelts of 2- to 3-month old female BALB/c mice (Jackson Labs, Bar Harbor, ME) as previously described [15]. Mice were killed by cervical dislocation and chemically depilated (Nair^R, Carter-Wallace Inc., New York, NY). Small sections of truncal skin (1 cm^2) were incubated dermal side down in cMEM (30 ml/pelt) containing 0.5% dispase and 2% FCS for 3 h at 37°C, 5% CO_2, 100% humidity. Epidermal sheets, lifted away from the dermis, were incubated an additional 60 min in cMEM containing 0.3% trypsin and 0.05% DNAse (10 ml/pelt) at 37°C, 5% CO_2, 100% humidity. After agitation, the EC suspension was filtered twice through nylon mesh (Nitex HC3-60, 60 nm pore size, Tetko Inc., Elmsford, NY) and centrifuged at 400 *g* for 10 min at 4°C. The cell pellet was resuspended and washed twice in cMEM/10% FCS. Approximately $4-5 \times 10^7$ EC per mouse pelt were obtained and viability ranged between 90 and 95%, as assessed by trypan blue dye exclusion.

Immunoabsorption Procedure to Enrich LC A solid phase immunoabsorption or "panning" procedure was employed as described by Rasanen et al [16], though with some modification. Briefly, 5-cm diameter petri plates were coated with 0.01% poly-L-lysine (mol wt 40,000; Sigma Chemical Co) for 45 min at room temperature, then washed 3 times with phosphate-buffered saline (PBS). Sheep red blood cells (SRBC) (2.5 ml of 1% suspension) were incubated on the plates for 45 min at room temperature, followed by 2 washes in PBS. Following overnight incubation at 4°C, 2.5 ml of a 1:200 dilution of rabbit-anti-SRBC IgG (Cappel Laboratories Inc., Cochranville, PA) was added. The plates were kept at 37°C for 45 min, then rinsed 3 times with PBS. 5×10^6 epidermal cells in cMEM/5% FCS (3 ml volume) were added to each plate and the plates centrifuged 5 min at 200 *g* to facilitate binding of LC to Fc portion of IgG, followed by incubation at 4°C for 2 h. The nonadherent cells were removed by 3 washes in PBS and SRBC lysed with 0.83% NH_4Cl-Tris buffer (pH 7.2). The LC-enriched population of cells was washed 3 times with cMEM/5% FCS and viability assessed by trypan blue dye exclusion. The cells were either used immediately in cell proliferation assay (below) or maintained in vitro in a 25-ml Wheaton stirring flash at 37°C, 5% CO_2, 100% humidity in cMEM/5% FCS.

Immunogold-Labeling Technique A postfixation immunogold-labeling technique, as described previously [15], was used to determine the degree of enrichment of LC in the panned-population of EC. The cell suspensions were labeled with monoclonal anti-Ia^d-antibody (anti-Ia^d, Becton Dickinson, Mountainview, CA). Following a wash in cMEM, cells were fixed in 1% paraformaldehyde-periodate lysine [17] 10 min at 4°C then washed in 0.1 M cacadylate buffer (pH 7.2). The presence of specifically fixed murine antibodies was revealed by incubation with protein A labeled with 15-nm colloid gold granules (pA-G15; Janssen Pharmaceutica, Beerse, Belgium) 1 h at 4°C. The colloid gold conjugate was diluted 1:4 in PBS containing 1% bovine serum albumin (PBS-BSA, pH 7.4, 50 μl/10^6 cells). After washing in 0.1 M cacadylate buffer, cells were postfixed first in 2% glutaraldehyde 30 min, then in 2% osmium tetroxide 20 min at 4°C. Enriched and nonenriched epidermal cell populations incubated in PBS/BSA alone (no colloid gold) or with cMEM alone (no primary antibody) served as negative controls for this series of experiments.

Electron Microscopy Immunogold-labeled cells prepared above were dehydrated through alcohol and embedded in epon-araldite. Sections (0.9 μm) stained with uranyl acetate and lead citrate were observed under a Phillips 301 transmission electron microscope. Langerhans cells were identified as those cells labeled with gold particles. Since a percentage of LC are Ia-negative, identifiable morphological features included a highly convoluted nucleus with intracytoplasmic Birbeck granules, an absence of keratin filaments, and a phase-lucent cytoplasm.

Preparation of Splenic T Lymphocytes Splenic T lymphocytes were obtained from 2- to 3-month old female mice (Jackson Labs) by a nylon-wool column purification technique described by Julius et al [18]. BALB/c mice (H-2^d haplotype) were used for the antigen-specific assay and CBA mice (H-2^k haplotype) were used for the allogenic assay. In the case of the antigen-specific cell proliferation assay, spleen lymphocytes were primed in vivo by inoculating mice i.p. with 250 μg ovalbumin in complete Freund's adjuvant 10 days before the assay. Briefly, the spleen was harvested, homogenized in Hanks balanced salt solution, and red cells lysed with 0.83% Tris-NH_4Cl (pH 7.2). After 3 washes in RPMI culture medium (RPMI-1640, Gibco; supplemented with 2 mM L-glutamine, 100 mM HEPES buffer, 100 U/ml penicillin G, 100 μg/ml Streptomycin, 5 mg/ml Fungizone, 5% heat-inactivated horse serum, and 5×10^{-3} 2-mercaptoethanol), the cells were percolated twice through nylon-wool columns (200×10^6 cells in 3 ml medium per 6 g nylon wool) for 60 min each. T cells were obtained by collecting the first 15 ml of eluate after addition of 15 ml of warm medium to the column. T cells were then washed repeatedly in RPMI culture media and resuspended to 2×10^6/ml.

Antigen-Specific (ovalbumin) Cell Proliferation Assay A secondary autologous antigen-specific cell proliferation assay as described by Alkan was used with modification [19]. Panned-enriched LC or crude EC suspensions at various times of incubation were pulsed in vitro with ovalbumin (1 mg/ml) 1 h at 37°C. The cells were then irradiated with 2500 r from 137Cs source, followed by 3 washes in RPMI culture medium. The cells were resuspended to a concentration of 5×10^4/ml (panned purified LC) or 5×10^6/ml (crude EC), and 100 μl of cell suspension was co-cultured with 100 μl of primed T lymphocytes (2×10^5/well) in triplicate 96-well flat bottom plates (Costar, Cambridge, MA) in RPMI culture medium. At 72 h, each well was pulsed with 2 μCi [^{3}H]methyl thymidine (ICN Biomedicals, Inc., Irvine, CA; 73 Ci/mMole). Cells were harvested 18 h later on a Mash II harvester and counted 2 min in a beta-scintillation counter. Negative control wells included T lymphocytes alone, and use of Langerhans cells or epithelial cells not pulsed with ovalbumin. Positive controls consisted of Conconavalin A (20 μg/ml) incubated with whole spleen cells.

Allogenic Cell Proliferation Assay A unidirectional mixed LC- or EC-lymphocyte proliferation assay was employed as described by Meo with modification [20]. Panned-enriched LC or crude EC (BALB/c origin) at different times of incubation were irradiated as described above, washed, then co-cultured with spleen T lymphocytes from CBA mice (H-2^k haplotype) in triplicate 96-well flat bottom plates for 6 days at 37°C, 5% CO_2, 100% humidity in RPMI culture medium. Each well contained 5×10^3 panned purified LC or 5×10^5 crude EC as stimulator cells and 2×10^5 splenic T cells. Each well was pulsed with [^{3}H]thymidine at 6 days and harvested 18 h later as described above. Negative controls consisted of LC or EC from BALB/c mice co-cultured with spleen T lymphocytes from BALB/c mice.

Back-Scattered Scanning Electron Imaging Back-scattered electron imaging was performed according to the procedure described by DeHarven and Solige [21]. Panned-enriched LC (50 μl of

cell suspension at 2×10^6 cells/ml) at 0 and 72 hours of incubation were attached to glass coverslips coated with poly-L-lysine (mol wt 70,000; Sigma Chemical Co) and fixed in buffered 0.2% glutaraldehyde for 5 min. Following rinsing in PBS containing 0.1% glycine, the cells were incubated with anti-Ia^d (1 : 10 dilution in PBS/BSA) for 30 min at room temperature, washed, then incubated 30 min with goat antimouse IgG conjugated to 40-nm colloid gold granules (GAM-G40, Janssen Pharmaceutica, Beerse, Belgium; 1 : 4 dilution in PBS/BSA). Cells were postfixed in 2% glutaraldehyde and prepared for scanning electron microscopy (SEM) by critical point-drying in CO_2 and conductive coating with carbon. Cells were examined by the SEM in the back-scattered imaging mode under 20 to 30 kV using a tungsten filament. The BE signal was mixed with the SE signal carrying the image of the cell surface structures. A total of 20 to 25 Langerhans cells were counted in different experiments to arrive at a mean number of gold particles per cell, which served as the parameter for surface density of Ia molecules.

RESULTS

Enrichment of LC by immunoabsorption (panning) yielded an 85% pure population of LC as assessed by immunoelectron microscopy with a viability consistently 95%.

Antigen-Specific Lymphocyte Proliferation Assay (Secondary Autologous) The results of the antigen-specific proliferation assay are illustrated in Fig 1. Results of four experiments at each time of incubation (0, 24, 48, and 72 h) are expressed as $\Delta\overline{CPM}$ (mean counts per minute using stimulator cells pulsed with ovalbumin, minus mean counts per minute using stimulator cells not pulsed with ovalbumin.) At time $t = 0$, LC in both panned-enriched or crude EC suspensions were relatively weak accessory cells compared to time $t = 72$ hours. For crude EC suspensions, there is a sixfold increase in the proliferation response of T lymphocytes; while only a 2.5-fold increase in the proliferative response was demonstrable with panned-enriched LC.

Allogenic Lymphocytes Proliferation Assay (Primary Allogenic) The results of the primary allogenic proliferation assay are illustrated in Fig 2. Results of four experiments at each time of culture (0, 24, 48, and 72 h) are expressed as $\Delta\overline{CPM}$ (mean counts per minute using stimulator cells from BALB/c mice and responder cells from CBA mice, minus mean counts per minute using stimulator and responder cells from BALB/c mice). At time $t = 0$, LC in both the panned-enriched and the crude EC suspensions are poor stimulator cells in this allogenic assay; whereas LC at $t = 72$ hours are strong accessory cells capable of inducing a strong primary allo-

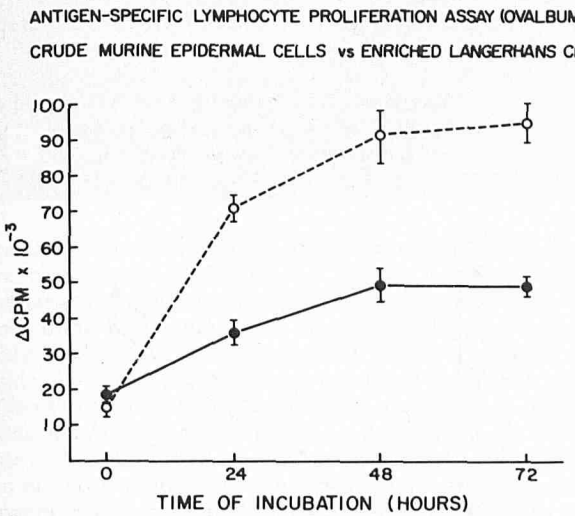

Figure 1. Ability of murine Langerhans cells (LC) maintained in vitro in short-term incubation to function as stimulator cells in a secondary autologous antigen-specific lymphocyte proliferation assay. Langerhans cells in a crude epidermal cell suspension (1% LC) (open circles) are compared to LC in a panned-enriched cell suspension (85% LC) (closed circles). Results of four experiments at each time (0, 24, 48, and 72 h of culture) are expressed as $\Delta\overline{CPM}$ (mean counts per minute using stimulator cells pulsed with ovalbumin minus CPM using stimulator cells not pulsed with antigen). Freshly prepared LC are weak accessory cells in this secondary response. There is a sixfold increase in the stimulatory capacity of LC in a crude EC suspension by 72 h, whereas only a 2.5-fold increase in functional ability is associated with panned-enriched LC.

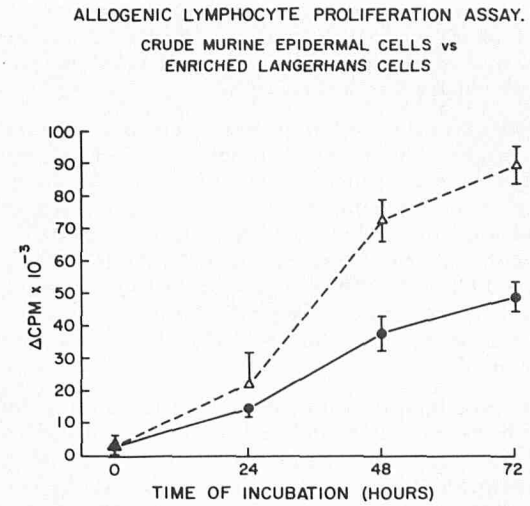

Figure 2. Ability of murine LC maintained in vitro in short-term incubation to function as stimulator cells in a primary allogenic proliferation assay. Langerhans cells in a crude epidermal cell suspension (1% LC) (open triangles) are compared to LC in a panned-enriched cell suspension (85% LC) (closed circles). Results of four experiments at each time (0, 24, 48, 72 h of culture) are expressed as $\Delta\overline{CPM}$ (mean counts per minute using stimulator cells from BALB/c mice [H-2^d] and T lymphocytes of CBA mice [H-2^k] minus CPM using stimulator cells and T lymphocytes from BALB/c mice). Freshly prepared LC are poor stimulators of a primary allogenic response. There is a 16- to 17-fold increase in ability of LC in a crude EC suspension to stimulate this response by 72 h, with a 12-fold increase in functional ability of LC in a panned-enriched suspension.

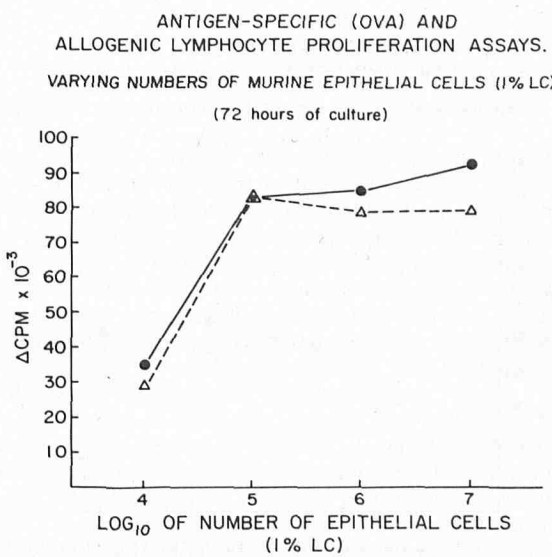

Figure 3. Effect of varying the number of 72 h–incubated epidermal cells (EC) on the secondary autologous (closed circles) and the primary allogenic (open triangles) assays. At least 5×10^5 crude EC are needed to obtain an optimal proliferation response. Increasing the number of EC 100-fold above this threshold has little effect on the proliferative response, which tends to plateau at and above the threshold number.

genic proliferation response. Using crude EC suspensions, there is a 16 to 17-fold increase in the stimulatory effect of 72-h cultured LC compared to freshly prepared LC; while a 12-fold increase is observed with use of panned-enriched LC.

Varying the Number of Langerhans Cells in the Cell Proliferation Assays The increased stimulatory effect observed with crude EC compared to panned-enriched LC can not be attributed to differences in absolute numbers of LC in the various assays (Figs 3 and 4). A minimum threshold number of LC is required to induce an optimal proliferative response. At least 1×10^5 crude EC (approximately 1% LC) or 1×10^3 panned-enriched LC are needed. Increasing the number of LC in each case 100-fold had little effect on the proliferative response, which tended to plateau at and above the threshold number.

Serum Is Not Responsible for Maturation of LC In Vitro When panned-enriched LC and crude EC were incubated in serum-free media, an increase in the stimulatory ability of LC by 72 h was still demonstrable (Figs 5 and 6), indicating that factor(s) responsible for maturation of LC in vitro is (are) either not limited to or present in serum. There is however a slight reduction in the stimulatory capabilities of LC maintained in vitro in serum-free media (15 to 30% reduction), which parallels an overall decrease in cell viability by 72 h (70 to 80% viability).

Maturation of LC In Vitro Is Not Due to Soluble Factors Released by Keratinocytes When cell-free medium from 72 h–incubated EC suspensions was used as the incubation medium for panned-enriched LC for 72 h, there was no enhancement of proliferative responses in either assay (data not shown). This finding indicates that keratinocyte-derived soluble factor(s) do not have a direct effect on LC maturation during incubation in vitro, and cannot explain the enhanced proliferative response observed using crude EC compared to panned-enriched LC (Figs 1 and 2). This finding, however, does not rule out some possible participation of certain factors derived from keratinocytes in the maturation of LC.

Surface Density of MHC-Class II Molecules Increase Three- to Fourfold by 72 Hours of Incubation In Vitro Using BEI, changes in surface density of MHC-class II molecules (Ia^d) were determined on immunogold-labeled LC in both panned-enriched and crude EC suspensions (Fig 7). At $t = 0$, the mean number of gold particles (gp) per cell was 70 (range 65–95); however, by $t =$ 72 h, the density increased to a mean of 260 gp/cell (range 200–290), representing a three- to fourfold increase. No difference between LC in the panned-enriched or crude EC suspension was noted. This three- to fourfold increase in surface expression of MHC-class II molecules parallels the increased functional ability of LC in culture.

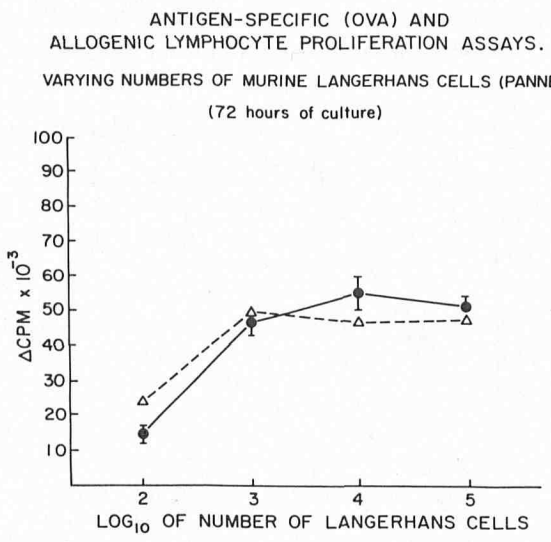

Figure 4. Effect of varying the number of 72 h–incubated panned-enriched LC on the secondary autologous (closed circles) and primary allogenic (open triangles) assays. At least 1×10^3 LC are needed to obtain an optimal proliferative response; increasing the number of LC 100-fold above this threshold has little effect on the proliferative response, which tends to plateau at and above the threshold number.

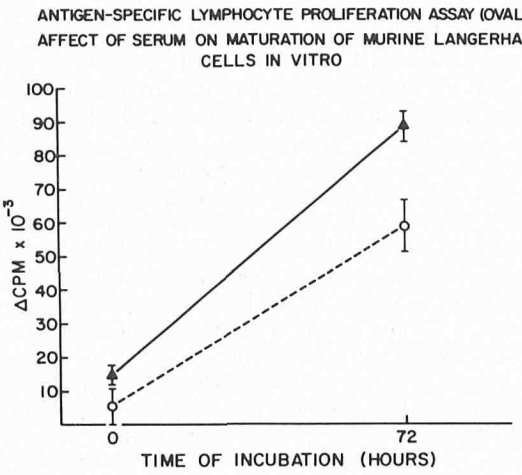

Figure 5. Effect of serum-supplemented (closed triangles) and serum-free (open circles) medium on a secondary autologous proliferation response using crude EC as stimulator cells. With both serum-supplemented and serum-free medium, there is an increase in the functional ability of crude EC to illicit a proliferative response. Serum-free medium, however, is associated with a 30% less increase, presumably due to an overall decrease in LC viability (70–80%). (* crude epidermal cells = 1% LC)

DISCUSSION

The results of our study indicate that LC mature into potent immunostimulatory cells through 72 h of incubation. They become 12- to 16-fold more potent as stimulator cells in a primary allogenic lymphocyte proliferation assay, compared to freshly prepared LC

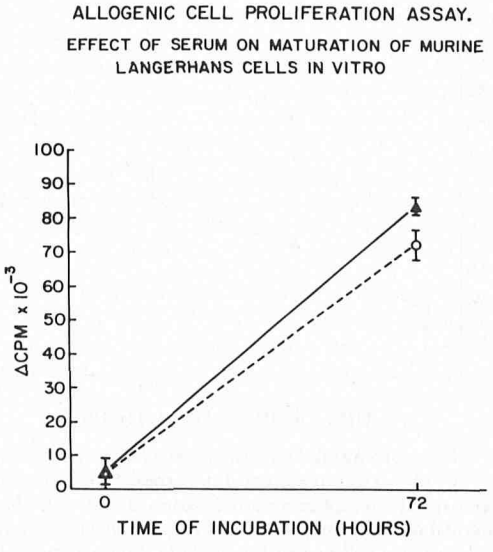

Figure 6. Effect of serum-supplemented (closed triangles) and serum-free (open circles) medium on a primary allogenic proliferation response using crude EC as stimulator cells. With both serum-supplemented and serum-free medium, there is an increase in the functional ability of crude EC to effect this primary response. Serum-free medium, however, is associated with 15% less increase, presumably due to an overall decrease in LC viability (70–80%). (* crude epidermal cells = 1% LC)

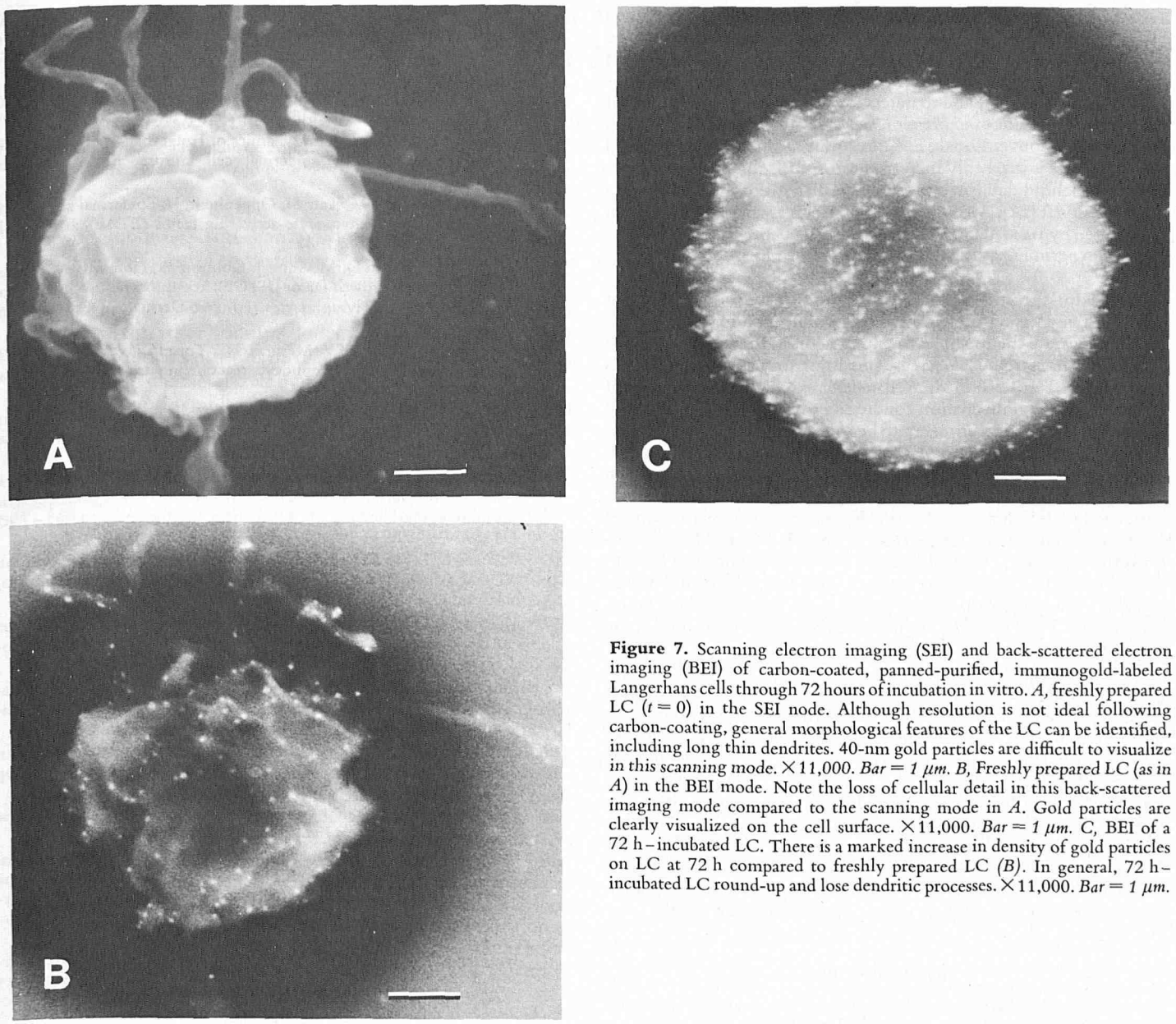

Figure 7. Scanning electron imaging (SEI) and back-scattered electron imaging (BEI) of carbon-coated, panned-purified, immunogold-labeled Langerhans cells through 72 hours of incubation in vitro. *A,* freshly prepared LC ($t = 0$) in the SEI node. Although resolution is not ideal following carbon-coating, general morphological features of the LC can be identified, including long thin dendrites. 40-nm gold particles are difficult to visualize in this scanning mode. ×11,000. *Bar = 1 μm. B,* Freshly prepared LC (as in *A*) in the BEI mode. Note the loss of cellular detail in this back-scattered imaging mode compared to the scanning mode in *A*. Gold particles are clearly visualized on the cell surface. ×11,000. *Bar = 1 μm. C,* BEI of a 72 h–incubated LC. There is a marked increase in density of gold particles on LC at 72 h compared to freshly prepared LC *(B)*. In general, 72 h–incubated LC round-up and lose dendritic processes. ×11,000. *Bar = 1 μm.*

that are very poor stimulators of this primary response. Furthermore, they acquire a 2.5- to 6-fold increase in ability to induce a secondary autologous antigen-specific lymphocyte proliferation response using ovalbumin as the foreign antigen. In comparison, freshly prepared LC are relatively weak accessory cells, yet are still capable of illiciting this secondary response. These results support a number of previous studies [7–9]. Schuler and Steinman [7], using murine EC partially enriched for LC, demonstrated a 30-fold increase in the ability of 72 h–incubated LC to induce T cell proliferation in a primary mixed lymphocyte response (MLR) or oxidative mitogenesis assay compared to freshly prepared LC. Inaba et al [8] studied the function of highly purified LC maintained in vitro for short term. They purified LC by flow cytometry after various times of incubation in a crude EC suspension. Langerhans cells purified after 12 h of culture were capable of stimulating a secondary antigen-specific and allogenic proliferation response but not a primary response; whereas 72 h of culture enabled the purified LC to stimulate both primary and secondary responses [8]. The reason freshly prepared LC are poor stimulators of a primary response is unclear. Inaba et al [8] suggested it is due to the inability of LC to promote T cell clustering by an antigen-independent method.

Although experimental evidence supports the fact that freshly prepared murine LC are poor stimulators of a primary proliferation response, this does not seem to be true of human LC. Rasanen et al [22] demonstrated that freshly prepared (82% purified) human LC are capable of a primary autologous and allogenic MLR. Demiden et al [23] also reported that freshly prepared human LC are quite capable of stimulating allogenic lymphocytes in a primary mixed skin cell-lymphocyte response, with a loss of this ability by 2 to 3 days of culture. These differences between dynamics of human and murine LC may represent species differences or the use of different responder cell lines in the functional assays employed.

The results of our study also demonstrate a three- to fourfold increase in surface expression of MHC-class II (Ia^d) molecules over 72 h of incubation in vitro. This is in agreement with Schuler and Steinman [7], who reported a twofold increase in Ia expression on murine LC maintained in vitro by measuring fluorescent intensity with flow cytometry. Human LC undergo a similar process of maturation in vitro, as do murine LC, in that they too increase expression of HLA-DR molecules [24]. The "poor" stimulatory abilities of freshly enriched LC cannot be explained by trauma affecting LC membranes during the enrichment procedure, since similar Ia densities were observed on LC within a crude EC suspension (before panning).

Back-scattered electron imaging of immunogold-labeled purified LC provides an advantage over flow cytometry and radioisotope binding assays to quantitate expression of antigen, since it provides the ability to directly visualize the surface expression and distribution of antigen per cell and to detect any possible subpopulations of LC regarding extent of expression of MHC-class II molecules [25]. As opposed to conventional SEM, the number of recognized labeled antigenic sites is markedly increased using BEI, since gold particles "hidden" behind dendrites or surface ruffles still transmit a detectable BEI signal [21]. Although gold particles as small as 20 nm can be detected by BEI [21], we preferred larger 40-nm particles for this series of experiments that resulted in more clear unambiguous labeling.

The factor(s) responsible for maturation of LC in vitro are not defined. The factor is not limited to serum, since maturation was observed despite the use of serum-free medium for incubation. We attributed the 15 to 30% decrease in stimulatory ability of the cells cultured in serum-free medium to decreased viability as a result of less than optimum incubation conditions. Soluble products released from keratinocytes do not seem to be solely responsible for maturation of LC in vitro for two reasons. First, panned-enriched LC still mature in vitro (in the virtual absence of KC), and cell-free medium from cultured KC do not enhance the maturation of panned-enriched LC. It is quite possible that KC participate in maturation of LC by cell-cell contact rather than by soluble factors. To help address this question, cell proliferation assays would need to be conducted using LC which were purified *after* 3 days of incubation in vitro in a crude EC suspension. Since LC in vitro lose their Fc-receptors after 2 to 3 days [7], our immunoabsorption procedure could not be performed at this time.

The increased stimulatory effect of crude EC compared to panned-enriched LC is most likely attributed to the enhanced effect of KC on T lymphocyte proliferation, perhaps through production of interleukin-1 or other stimulatory cytokines. Inaba et al [8] also found that purified LC were weaker stimulators than unseparated (crude) EC, suggesting that the presence of keratinocytes augmented the proliferation response in their MLR assay. It is also possible that KC and LC co-participate in processing and presenting foreign antigen to T lymphocytes, as suggested by Katz et al [3]. Lastly, one can not rule out the possibility that the T cell response is augmented by organ-specific alloantigens (non-MHC transplantation antigens such as EPA-1) on the keratinocyte [26–27].

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